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Abstract

Long term storage of *R. solani* was improved by using the freezing and thawing conditions that are generally used for animal/human cell lines. Key factors to success were the use of controlled freezing and the use of complete mycelial colonies.

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Long Term Storage of Isolates of *Rhizoctonia Solani*, a Mycelial Fungus

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Long term storage of *R. solani* was improved by using the freezing and thawing conditions that are generally used for animal/human cell lines. Key factors to success were the use of controlled freezing and the use of complete mycelial colonies.

The soil-borne plant pathogenic fungus *Rhizoctonia solani* is highly diverse and variable. For research purposes it is necessary to prevent genetic variability and to be able to maintain isolates for a long period of time in their original condition. Since *R. solani* does not make vegetative spores, these cannot be used for storage, which is usually done by maintenance at 4 C on e.g., Mouth-Pepton-Agar (MPA; Oxoid) or Potato-Dextrose-Agar (PDA; Difco) plates with periodical transfer to new plates. This method of storage is unsuitable for molecular studies since the isolates grow and may change and, furthermore, plates may become contaminated, dry out, etc. Alternative methods like dry storage on soil or soil-bran mixtures have been published (e.g., Butler EE (1980) *Phytopathology* 70:820-821), but work unsuccessfully in our hands. A significant percentage of isolates could not be recovered. In addition, we encountered contamination. Alternatively, isolates may be stored in liquid nitrogen in the presence of cryoprotectants. Storage at low temperatures is the method of choice for long term preservation of biological material and this option was therefore pursued.

Methods

We optimized storage of *R. solani* to the following conditions. Isolates were first grown on defined nutrient agar plus vitamins (Media contained the following per liter:15 g sucrose, 2 g asparagine, 0.6 g KH2PO4, 0.8 g K2HPO4, 1 g MgSO4, 10 mg CaCl2, 2.5 mg ZnSO4.7H2O, 2.5 mg H3BO4, 0.5 mg MnSO4.1H2O, 1.7 mg NaFe EDTA, 0.3 mg CuSO4.5H2O, 0.1 mg (NH4)6Mo7O24.4H2O, 1.0 mg thiamine, 1.0 mg niacin, 20 ug biotin, 0.5 mg Ca-pantothenate, 0.5 mg pyridoxine and 0.1 mg p-aminobenzoic acid and 2 g agar for solid medium) at 23 C in the dark. 3 mm3 of inoculum was transferred from the growing edge of a colony to 1 ml of liquid Potato Dextrose Medium (PDM, Difco) plus vitamins in a 24 well tissue culture plate and grown at 23 C in the dark until the well was just filled. The mycelium was then transferred to 1.5 ml 15 % (v/v) glycerol in a cryovial. The vial was placed in a Cryo 1C Freezing Container (Nalgene, Rochester, NY), with isopropanol as indicated by the supplier. Next, the container was placed at 4 C for 1-4 h and then transferred to -80 C for 4 - 24 h. Finally, the cryovials were transferred directly to liquid nitrogen for permanent storage. To recover the isolate, the vial was taken from the liquid nitrogen and warmed rapidly by shaking in a 37 C waterbath until it was partially thawed. The vial then was placed on ice and the mycelium was transferred from the vial to a fresh PDA plus vitamins plate, which was incubated at 23 C in the dark.

Most steps of the storage procedure were tested by using agar slants of a limited number of isolates, including some isolates that gave difficulties in recovery in the usual storage at 4 C. The optimized procedure was subsequently tested for 44 *R. solani* isolates belonging to AGs 1, 2-1, 2-t, 2-2, 3, 4, 5, 6, 8, 9, 10 and AG-BI. The recovery was tested in after 1 month and after 1 year, with identical results (Table 1).

Table 1. Low temperature storage of *Rhizoctonia solani* isolates: Influence of the sample preparation and the freezing procedure on the recovery.

SAMPLE	FREEZING	RECOVERY	
		YES	NO
Agar slant	stepwise	31 isolates (70 %)	13 isolates (30 %)
Agar slant	slow controlled	40 isolates (90 %)	4 isolates (10 %)
Mycelial colony	slow controlled	42 isolates (95 %)	2 isolates (5 %)

Cryoprotectant. (Tested for micro-slants (3 mm^3) with controlled freezing and fast thawing) 10 % DMSO (dimethylsulfoxide), 50 % glycerol and 15 % glycerol were compared. 15 % glycerol gave the best results (5 out of 5 recovered) and was the cryoprotectant of choice. The use of 50 % glycerol and 10 % DMSO resulted in no recovery using microslants (0 out of 5 recovered).

Sample size. (Tested with controlled freezing and fast thawing) Isolates could be recovered from 10 % DMSO (2 out of 3), 15 % glycerol (3 out of 3) and 50 % glycerol (3 out of 3) when 1 cm^2 slants were used, leading to the conclusion that larger slants gave better recovery than microslants (see above). In a larger experiment, the use of a mycelium colony instead of a 1 cm^2 slant resulted in further improvement (Table 1).

Freezing. (Tested with 15 % glycerol as cryoprotectant) Slow freezing at a controlled rate of 1 C per minute increased the recovery of *R. solani* isolates (Table 1). Controlled freezing can be done by using automated equipment. We used a cheaper alternative; the Nalgene Cryo 1 C freezing Container with isopropanol to moderate temperature reduction. Compared to freezing directly in liquid nitrogen (data not shown) or freezing in steps (2 h at -20 C, 2 h at -80 C, and then to liquid nitrogen), controlled freezing increased the recovery (Table 1).

Thawing. (Tested for slants with controlled freezing and 15 % glycerol) Thawing on ice, thawing at room temperature and thawing at 37 C were compared. Best was thawing at 37 C in a waterbath until the sample was almost completely thawed (7 out of 8 recovered isolates as compared to 5 out of 8 for the other two conditions).

Growth substratum. (Tested for mycelial colony with controlled freezing, fast thawing and 15 % glycerol). PDM/PDA plus vitamins, DNM/DNA plus vitamins and PDM/water-agar were used as growth substratum before and after storage. The combination of PDM and PDA (both with vitamins) gave the best results, the isolates grew out rapid and reproducibly. This is probably related with the higher mycelial density on this substratum as compared to the others, where growth started later, was sparse and did sometimes not occur (especially on water agar).

Final storage temperature. (Tested for mycelial colonies with controlled freezing, fast thawing and 15 % glycerol) After 1 month no difference in recovery was observed between storage at -80 C or in liquid nitrogen (tested for 7 isolates). Recoveries after extended storage periods were not compared.

Biological activity. No variation in biological activity was observed as a result of freezing and thawing. This was determined by analyzing the symptom development by 5 isolates on 4 different plant species (data not shown).

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